

JC07 Rec'd PCT/PTO 27 FEB 2002

Form PTO 1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV 5-93)		ATTORNEY'S DOCKET NUMBER P51032
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 10/070128
INTERNATIONAL APPLICATION NO PCT/US00/26949	INTERNATIONAL FILING DATE 29 September 2000	PRIORITY DATE CLAIMED 29 September 1999
TITLE OF INVENTION METHOD OF USING ONE-DIMENSIONAL AND MULTI-DIMENSIONAL NUCLEAR MAGNETIC RESONANCE TO IDENTIFY COMPOUNDS THAT INTERACT WITH TARGET BIOMOLECULES		
APPLICANT(S) FOR DO/EO/US Jacques BRIAND		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98; Form PTO-1449, and a copy of the International Search Report.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☒ Please amend the specification by inserting before the first line the sentence: This is a 371 of International Application PCT/US00/26949, filed 29 September 2000, which claims benefit from the following U.S. Provisional Application: 60/156,577, filed 29 September 1999.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ An Abstract on a separate sheet of paper.

JC19 Rec'd PCT/PTO 27 FEB 2002

19. [] Other items or information:

US APPLICATION NO. (if known see 37 CFR 1.50) 10/070128		INTERNATIONAL APPLICATION NO. PCT/US00/26949		ATTORNEYS DOCKET NO P51032	
20. [X] The following fees are submitted:				CALCULATIONS PTO USE ONLY	
Basic National Fee (37 C.F.R. 1.492(a)(1)-(5)):				\$710.00	
Search Report has been prepared by the EPO or JPO\$890.00					
International Preliminary Examination Fee paid to USPTO (37 CFR 1.492)\$710.00					
No International Preliminary Examination Fee paid to USPTO (37 CFR 1.492) but international search fee paid to USPTO (37 CFR 1.445(a)(2))\$740.00					
Neither International Preliminary Examination Fee (37 CFR 1.492) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$1,040.00					
International Preliminary Examination Fee paid to USPTO (37 CFR 1.492) and all claims satisfied provisions of PCT Article 33(2)-(4).....\$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$710.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$0.00	
Claims	Number Filed	Number Extra	Rate		
Total claims	19 - 20 =	0	0 x \$18.00	\$0.00	
Independent claims	4 - 3 =	1	1 x \$84.00	\$84.00	
Multiple dependent claims (if applicable)			+ \$280.00	\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$794.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$	
SUBTOTAL =				\$794.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				\$	
TOTAL NATIONAL FEE =				\$794.00	
				Amount to be refunded	\$
				charged	\$

- a. ☐ A check in the amount of \$_____ to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 19-2570 in the amount of **\$794.00** to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-2570. A duplicate copy of this sheet is enclosed.
- d. ☒ General Authorization to charge any and all fees under 37 CFR 1.16 or 1.17, including petitions for extension of time relating to this application (37 CFR 1.136 (a)(3)).

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

GLAXOSMITHKLINE

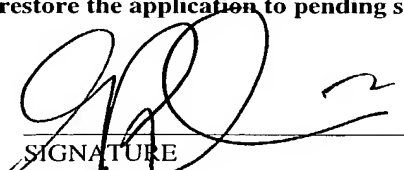
Corporate Intellectual Property - UW2220

P.O. Box 1539

King of Prussia, PA 19406-0939

Phone (610) 270-4478

Facsimile (610) 270-5090



SIGNATURE

Edward R. Gimmi

NAME

38,891

REGISTRATION NO.

METHOD OF USING ONE-DIMENSIONAL AND MULTI-DIMENSIONAL NUCLEAR
MAGNETIC RESONANCE TO IDENTIFY COMPOUNDS THAT INTERACT WITH
TARGET BIOMOLECULES

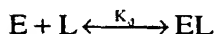
5

FIELD OF THE INVENTION

The present invention relates to a method for identifying and classifying
compounds that bind to target biomolecules using one- or two-dimensional nuclear
10 magnetic resonance (NMR) spectroscopy.

BACKGROUND OF THE INVENTION

The use of nuclear magnetic resonance for screening and detecting weakly binding
compounds to biomolecular targets has been recently described in patent documents
15 US5698401, US5804390, US5891643, DE19649359, PCT publications WO 98/48264, WO
98/57155 and other publications, Meyer et al. Eur.J.Biochem. 246 705-709 (1997), Lin et
al. J.Am.Chem.Soc. 119 5249-5250 (1997), Chen et al. J.Am.Chem.Soc. 120 10258-10259
(1998), Lin et al. J.Org.Chem. 62 8930-8931 (1997), Hajduk et al. J.Am.Chem.Soc. 119
12257-12261 (1997), Shuker et al. Science 274 1531-1537 (1996), Klein et al.
20 J.Am.Chem.Soc. 121 5336-5337 (1999), Mayer et al. Angew. Chem. (1999). There are
several NMR techniques covered in these publications and they all rely at various degrees
on observing changes in NMR chemical shifts, changes in resonance linewidths, changes in
translational diffusion, or changes in dipolar relaxation phenomena of either the target or
the ligand upon undergoing exchange between the bound and unbound state. The bound and
25 unbound populations are dictated by the affinity between the target molecule (E) and the
ligand (L) following the binding reaction at equilibrium



where K_d is the dissociation constant expressed by

$$K_d = \frac{[E][L]}{[EL]}$$

30 The bound (p_b) and unbound (p_u) populations are expressed are by

$$p_b = \frac{[L]}{K_d + [L]} \quad p_u = \frac{K_d}{K_d + [L]}$$

The greater the value of K_d , the smaller the affinity. It is well known in the art that if binding affinity is weak (K_d typically at sub-millimolar concentration or higher), the bound population can be small and at typical NMR ligand concentrations (submillimolar to millimolar), a relatively large target concentration (typically up to low submillimolar) is required for populating the bound state and observe perturbations on the NMR signal of the ligand. Inversely, if the NMR signal of the target molecule is being observed, a large ligand concentration is required for populating the target molecule and a relatively large concentration (typically submillimolar range) of the target molecule is also required in order to record a NMR spectrum with a reasonable S/N ratio. The latter also has the shortfall of being limited to relatively low molecular weight targets (< 20-35KDa) and requires isotopic labelling of the target. Thus, a major shortcoming of these existing NMR screening techniques for identifying weakly binding ligands is their necessity of having to use a high target concentration in order to perturb the NMR spectral parameters at detectable levels.

Clearly, there exists a need for a faster and less expensive method for identifying ligands to target biomolecules as this undergirds the pharmaceutical discovery process, among other things. Further, these methods have a present benefit of, among other things, being useful to screen compounds for biological activities. Such methods are also useful for determining the role of targets and such compounds in pathogenesis of infection, dysfunction and disease in individuals. There is also a need for methods for identification and characterization of such targets and their antagonists and agonists to find ways to prevent, ameliorate or correct such infection, dysfunction and disease in individuals. The present fulfils this unmet need by providing methods using one-dimensional and multi-dimensional NMR spectroscopy for identifying compounds that interact with target biomolecules.

SUMMARY OF THE INVENTION

The present invention relates to methods using one-dimensional and multi-dimensional NMR spectroscopy for identifying ligands to target biomolecules. One-dimensional and two-dimensional NMR spectroscopy are preferred in the methods of the invention. Moreover, methods are provided by the invention for using one-dimensional and multi-dimensional nuclear magnetic resonance to identify products, ligands or substrates of enzymatic target biomolecules.

WO 01/23889

PCT/US00/26949

A further embodiment of the invention provides a method for identifying compounds that bind to or interact with a specific target molecule. One preferred method comprises the steps of: a) mixing a substrate or product of a target biomolecule with one or a mixture of chemical compounds; b) generating a first one- or two-dimensional spectrum that displays either ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shifts in the first dimension or ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shifts in the other dimension of substrate or product in step a); c) exposing substrate or product and mixture of chemical compounds in step a) to a target molecule for one or more incubation times; d) generating a second first one- or two-dimensional spectrum that displays either ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shifts in the first dimension or ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shifts in the other dimension of substrate or product in step a) that has been exposed to the target molecule in step c) in the presence of one or mixture of chemical compounds in step a); e) comparing said first and second one- or two-dimensional NMR spectra after one or more said incubation times in step c) to determine differences between said first and second NMR spectra, the differences observed along either or both chemical shift dimensions identifying the transformation of said substrate classifying the presence of one or more compounds that are ligands which have bound to the enzymatic site of said target molecule.

Another embodiment of the invention is a method of identifying compounds that interact with a target molecule comprising the steps of: a) mixing a substrate, product or ligand of a target with at least one chemical compounds; b) generating a first spectrum that displays either a chemical shift in the first dimension or a chemical shifts in the other dimension of substrate, product or ligand in step a); c) exposing substrate, product or ligand and mixture of chemical compounds in step a) to a target molecule for one or more incubation times; d) generating a second spectrum that displays either a chemical shifts in the first dimension or a chemical shifts in the other dimension of substrate or product in step a) that has been exposed to the target molecule in step c) in the presence of one or mixture of chemical compounds in step a); e) comparing said first spectrum and second spectrum after one or more said incubation times in step c) to determine at least one difference between said first spectrum and second spectrum, the differences observed along either or both chemical shift dimensions identifying the transformation of said substrate and classifying the presence of one or more compounds that are substrates, products or ligands that interact with said target molecule.

Still another embodiment of the invention provides a method of identifying compounds that interact with a target molecule comprising the steps of: a) exposing

substrate to a target molecule for one or more incubation times; b) generating one or more spectra at one or more incubation times of said substrate and said target molecule of step a), c) exposing said substrate and one or mixture of chemical compounds for one or more incubation times; d) generating one or more spectra at one or more incubation times of said substrate, said target molecule and said compounds of step c); e) comparing at least one spectrum of step b) with at least one spectrum of step d) to determine at least one difference between said spectrum of step b) with said spectrum of step d), the differences observed along either or both chemical shift dimensions identifying the transformation of said substrate and classifying the presence of one or more compounds that are substrates, products or ligands that interact with said target molecule.

The invention also provides a method wherein step a) further comprises a target that is a biomolecule.

The invention still further provides a method wherein step a) further comprises a chemical compound that is in solution or attached to a solid substrate or matrix.

Another embodiment of the invention is a method wherein step b) further comprises a first spectrum selected from the group consisting of a one-dimensional, two-dimensional or three-dimensional spectrum, and/or wherein said first spectrum displays a chemical shift in said first dimension selected from the group consisting of a ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift, and a chemical shift in said other dimension selected from the group consisting of ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift.

A further embodiment of the invention provides a method wherein said exposing step of step c) further comprises a mixture comprising between 2 and 100 chemical compounds, and/or wherein said incubation times number between 1 and 20, 30, 40 50 or greater.

A still further embodiment of the invention provides a method of claim 1 wherein step d) said second spectrum displays a chemical shift in said first dimension selected from the group consisting of a ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift, and a chemical shift in said other dimension selected from the group consisting of ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift.

Also provided by the invention is a method wherein said comparing step of step e) comprises comparing first one- or two-dimensional NMR spectra and second one- or two-dimensional NMR spectra after one or more said incubation times.

A method is provided wherein the determining step of step e) comprises a method selected from the group consisting of an algorithm, a computer algorithm, and visual inspection.

Another method of the invention provides a method wherein an interaction is selected from the group consisting of molecule-molecule binding, ligands bound to an enzymatic site of said target molecule.

A preferred embodiment is a method of determining an interaction constant (α) comprising the steps of: a) exposing a substrate or product to a target molecule for one or more incubation times; b) generating a first spectrum that displays either a chemical shift in the first dimension or a chemical shifts in an other dimension of the substrate or product in step a) that has been exposed to the target molecule; c) mixing a substrate or product with the first ligand; d) exposing the substrate or product and the first ligand to the target molecule for one or more incubation times; e) generating a second spectrum that displays either a chemical shift in the first dimension or a chemical shift in the other dimension of substrate or product in step c) that has been exposed to the target molecule in step d) in the presence of the first ligand in step c); f) mixing the substrate or product with one or more chemical compounds; g) exposing the substrate or product and one or more chemical compounds to a target molecule for one or more incubation times; h) generating a third spectrum that displays either a chemical shift in the first dimension or a chemical shift in the other dimension of substrate or product in step f) that has been exposed to the target molecule in step g) in the presence of the one or more chemical compounds in step f); i) mixing substrate or product with first ligand and one or more chemical compounds; j) exposing the substrate or product, the first ligand and the one or more chemical compounds to the target molecule for one or more incubation times; k) generating a fourth spectrum that displays either a chemical shift in the first dimension or a chemical shift in the other dimension of the substrate or product in step i) that has been exposed to the target molecule in step j) in the presence of the first ligand and the one or more chemical compounds in step f); l) determining a conversion rate or conversion rates of each substrate or product from each spectrum of steps b), e) ,h) and k); and deriving an interaction constant (α) from a steady-state rate equation.

A method using NMR for screening for ligands which exhibit synergistic effects on a target in the presence of another ligand is also provided by the invention.

A method wherein the target is an enzyme comprising more than two binding sites is further provided, including, for example a binding site comprising a proximal and/or integral sub-binding site or sites.

Also provided is a method wherein the two binding sites are a substrate- and co-enzyme-binding site.

A method wherein the rate is determined by the following equation:

$$v = V_m / \left[1 + \left(\frac{K_M}{S} \right) \left(1 + \frac{I_1}{K_{EI1}} + \frac{I_2}{K_{EI2}} + \frac{I_1 I_2}{\alpha K_{EI1} K_{EI2}} \right) \right]$$

wherein S, I₁ and I₂ are the substrate, inhibitor I₁ and inhibitor I₂ concentrations, respectively.

- 5 Further methods are provided wherein said at least one chemical compound is provided in a multiwell vessel loaded with target and ligand and/or substrate and/or product.

Methods are also provided wherein a target-substrate reaction is quenched at a selected time to stop the reaction.

- 10 Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following descriptions and from reading the other parts of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

- 15 Figure 1 depicts the deformylation of For-Met-Ala-Ser-OH catalyzed by the Ni²⁺ form of Peptide Deformylase (*S. Aureus*) (herein also referred to as "PDF") over a range of incubation times, as monitored by 1H nuclear magnetic resonance spectroscopy. The top row is a control experiment depicting the stability of For-Met-Ala-Ser-OH. Only the resonance peak of the amide proton of the Ser residue is shown over the time course. In the middle row, For-Met-Ala-Ser-OH (S: substrate) is deformylated by Ni-PDF and its transformation into Met-Ala-Ser-OH (P: product) is monitored through the appearance of a second resonance peak, labelled as "P" at the left of the original resonance peak labelled as "S". In the bottom row, 8-hydroxyquinoline is added to the mixture and the deformylation of For-Met-Ala-Ser-OH is slightly inhibited, as seen by the decreased rate at which the resonance peak "P" increases over the time course.
- 20
- 25

- Staphylococcus aureus Peptide Deformylase is described in U.S. Patent Application Serial Number 08/911,844, filed August 15, 1997, and European Patent Applications EP008798879A2 and EP00879879A3. The methods of the example could also be adapted for use with Streptococcus pneumoniae pdf1 (U.S. Patent Application Serial Number 08/991,023, filed December 15, 1997 and European Patent Applications EP00863152A3 and EP00863152A2) or Streptococcus pneumoniae pdf2 (U.S. Patent Application Serial
- 30

Number 08/989,558, filed December 12, 1997 and European Patent Application EP00863205A1).

Figure 2 follows the same description as Figure 1 with the only difference being that the resonance peak of the formate group is monitored over the same time course.

5 Formate is a by-product of the deformylation of For-Met-Ala-Ser-OH.

Figure 3 depicts the reaction scheme when two substrate-competitive inhibitors (I_1 and I_2) and a substrate (S) react with an enzyme (E). It is assumed that I_1I_2 , I_1S , I_2S , I_1I_2S , EI_1S , EI_2S and EI_1I_2S are not formed in this system. An interaction constant α between I_1 and I_2 in the EI_1I_2 complex has the following meaning: when I_1 and I_2 interact with the same site of E , they prevent each other from binding to E , i.e. EI_1I_2 is not formed and thus $\alpha = \infty$. If I_1 and I_2 interact with different sites of E , $\infty > \alpha > 0$. When the interactions of I_1 and I_2 with E are strictly independent of each other, $\alpha = 1$. When a positive attraction occurs or if synergy exists between the two inhibitors in the EI_1I_2 complex, $1 > \alpha > 0$. When I_1 and I_2 interact repulsively in the EI_1I_2 complex, $\infty > \alpha > 1$.

15 Figure 4 depicts the deformylation of For-Met-Ala-Ser-OH catalyzed by the Ni^{2+} form of Peptide Deformylase (*S.Aureus*) over a range of incubation times, as monitored by 1H nuclear magnetic resonance spectroscopy of the buildup of the formate (For) moiety. Four slopes are illustrated in this figure, the first one (\circ) is a "control" experiment using 2.5mM of substrate (For-Met-Ala-Ser-OH) and 1uM of enzyme. The second slope (\square) contains 0.4mM of 8-hydroxyquinoline (I_1) in the presence of 2.5mM of substrate and 1uM of enzyme. The third slope (∇) contains 0.15mM of N-Nitro-N'-(N-phenylamino)guanidine (I_2) in the presence of 2.5mM of substrate and 1uM of enzyme. Finally the fourth slope (\diamond) contains both I_1 and I_2 at 0.4mM and 0.15mM, respectively, in the presence of 2.5mM of substrate and 1uM of enzyme. Calculating the four slopes and using the steady-state rate equations for this system, it is straightforward to calculate the K_{EI1} , K_{EI2} , and α value. Assuming a K_M of 2.5mM, we get 335uM, 151uM and 0.72, for K_{EI1} , K_{EI2} , and α , respectively. This example demonstrates that it is possible to screen for a second inhibitor I_2 in the presence of a first inhibitor I_1 and determine whether they interact in a highly desirable synergistic fashion, or whether they interact repulsively or compete for the same site.

30 Figure 5 depicts the deformylation of For-Met-Ala-Ser-OH catalyzed by the Ni^{2+} form of Peptide Deformylase (*S.Aureus*) over a range of incubation times, as monitored by 1H nuclear magnetic resonance spectroscopy. Only the resonance peak of the amide proton of the Ser residue is shown over the time course. In the top row, For-Met-Ala-Ser-OH (S :

WO 01/23889

PCT/US00/26949

substrate) is deformylated by Ni-PDF and its transformation into Met-Ala-Ser-OH (P: product) is monitored through the appearance of a second resonance peak, labelled as "P" at the left of the original resonance peak labelled as "S". In the bottom row, the same experiment as in the top row is performed but at an incubation time of about 22-24 minutes, the enzymatic reaction is quenched by adding hydrazine (15mM final sample concentration) to the sample. Following this procedure, the deformylation of For-Met-Ala-Ser-OH is further monitored by ¹H nuclear magnetic resonance spectroscopy over a time course and it can be seen that the enzymatic reaction has been successfully stopped, as judged from the lack of progression of the resonance peaks labelled as "S" and "P".

Figure 6 illustrates further examples demonstrating that it is possible to screen for a second inhibitor I₂ in the presence of a first inhibitor I₁ and determine whether they interact in a highly desirable synergistic fashion, or whether they interact repulsively or compete for the same site. In the specific examples shown here, the Yonetani-Theorell graphical method is used for determining the interaction constant "α" between two inhibitors. In this method, enzyme kinetic rates, v, are measured at fixed concentrations of substrate and in the presence of variable concentrations of two inhibitors I₁ and I₂. First, a set of lines is obtained for 1/v versus [I₁] for a series of fixed [I₂]. The abscissa at the point of intersection of this set of lines corresponds to -αK_{EI1}, where K_{EI1} is the constant of inhibition for inhibitor I₁. K_{EI1} is also graphically determined using the Dixon graphical method : a family of lines for 1/v versus [I₁] for a series of fixed [S] is obtained and the abscissa at the point of intersection of this family of lines corresponds to -K_{EI1}. In a) and b), two pairs of ligands are shown to interact synergistically with α < 1, suggesting that they can bind simultaneously in a synergistic fashion.. Inversely in c), formohydroxamic acid and 8-hydroxyquinoline are shown to compete for the same subsite with α >> 1, suggesting that they interact repulsively and cannot bind simultaneously with the enzyme.

DESCRIPTION OF THE INVENTION

An embodiment of the present invention provides a target molecule involved in a catalytic reaction of a substrate into a final product. Not being limited in any way by a theoretical or mathematical model, a simple example of such a process may be described, among other ways, by the following reaction mechanism



where the target molecule is an enzyme (E) which catalyzes the conversion of a substrate "S" into a product "P". The velocity of the enzyme-catalyzed reaction, v , can be expressed as a function of substrate and enzyme concentration by the well-known Michaelis-Menten equation

5
$$v = \frac{k_{\text{cat}}[S][E_0]}{K_M + [S]}$$

where $[E_0]$ is the total enzyme concentration, $[S]$ is the substrate concentration and the ratio k_{cat}/K_M is a measure of the catalytic efficiency of the enzyme for a particular substrate. It is well-known in the art of enzymology, that, although there can be a wide range of catalytic efficiencies for various substrates of a particular enzyme, this catalytic efficiency can be
10 extreme under favorable condition up to diffusion limits of about $10^9 \text{ s}^{-1}\text{M}^{-1}$.

In a preferred embodiment of the present invention, this catalytic efficiency is exploited in order to decrease by several orders of magnitude the amount of target molecule required to detect binding of a compound to the target molecule. This can be done by monitoring changes in the conversion rate of a substrate into a product when the compound
15 is present.

Another preferred embodiment provides a method wherein changes observed in a parameter of an enzymatic reaction, such as reaction rate can be measured. Example 1 provides on such method. For target enzymes having catalytic efficiencies in the range of $10^5 \text{ s}^{-1}\text{M}^{-1}$ for that substrate, it is preferable to use a target concentration which is at least
20 two orders of magnitude smaller than the concentration that would have been required for identifying a known substrate as a ligand by any known NMR technique, such as the well-known technique of differential line broadening.

Another advantage of the methods of the invention for weakly binding ligands, is that an NMR spectrum also contains their fingerprint which can be used to monitor their
25 stability simultaneously with the substrate conversion.

Another advantage compared to coupled assays is to reduce the number of false positives found in coupled assays which rely on additional reagents which can potentially react with the screened compounds downstream or upstream and appear as if the compound was interacting with the target of interest. In the present invention, there is no need for
30 these additional reagents since the substrate is directly observed by NMR.

A further embodiment of the invention provides method for identifying compounds that bind to or interact with a specific target molecule. One preferred method comprises the steps of: a) mixing a substrate or product of a target biomolecule with one or a mixture of

chemical compounds; b) generating a first one- or two-dimensional spectrum that displays either ¹H, ³H, ¹¹B, ¹³C, ¹⁵N, ¹⁹F, ²⁹S or ³¹P chemical shifts in the first dimension ¹H, ³H, ¹¹B, ¹³C, ¹⁵N, ¹⁹F, ²⁹S or ³¹P chemical shifts in the other dimension of substrate or product in step a); c) exposing substrate or product and mixture of chemical compounds in step a) to a target molecule for one or more incubation times; d) generating a second first one- or two-dimensional spectrum that displays either ¹H, ³H, ¹¹B, ¹³C, ¹⁵N, ¹⁹F, ²⁹S or ³¹P chemical shifts in the first dimension or ¹H, ³H, ¹¹B, ¹³C, ¹⁵N, ¹⁹F, ²⁹S or ³¹P chemical shifts in the other dimension of substrate or product in step a) that has been exposed to the target molecule in step c) in the presence of one or mixture of chemical compounds in step a); e) comparing said first and second one- or two-dimensional NMR spectra after one or more said incubation times in step c) to determine differences between said first and second NMR spectra, the differences observed along either or both chemical shift dimensions identifying the transformation of said substrate or product and classifying the presence of one or more compounds that are ligands which have bound to the enzymatic site of said target molecule.

Another embodiment of the invention is a method of identifying compounds that interact with a target molecule comprising the steps of: a) mixing a substrate, product or ligand of a target with at least one chemical compounds; b) generating a first spectrum that displays either a chemical shift in the first dimension or a chemical shifts in the other dimension of substrate, product or ligand in step a); c) exposing substrate, product or ligand and mixture of chemical compounds in step a) to a target molecule for one or more incubation times; d) generating a second spectrum that displays either a chemical shifts in the first dimension or a chemical shifts in the other dimension of substrate or product in step a) that has been exposed to the target molecule in step c) in the presence of one or mixture of chemical compounds in step a); e) comparing said first spectrum and second spectrum after one or more said incubation times in step c) to determine at least one difference between said first spectrum and second spectrum, the differences observed along either or both chemical shift dimensions identifying the transformation of said substrate and classifying the presence of one or more compounds that are substrates, products or ligands that interact with said target molecule.

The invention also provides a method wherein step a) further comprises a target that is a biomolecule.

The invention still further provides a method wherein step a) further comprises a chemical compound that is in solution or attached to a solid substrate or matrix.

Another embodiment of the invention is a method wherein step b) further comprises a first spectrum selected from the group consisting of a one-dimensional, two-dimensional or three-dimensional spectrum, and/or wherein said first spectrum displays a chemical shift in said first dimension selected from the group consisting of ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift, and a chemical shift in said other dimension selected from the group consisting of ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift.

A further embodiment of the invention provides a method wherein said exposing step of step c) further comprises a mixture comprising between 2 and 100 chemical compounds, and/or wherein said incubation times number between 1 and 20, 30, 40, 50 or greater.

A still further embodiment of the invention provides a method of claim 1 wherein step d) said second spectrum displays a chemical shift in said first dimension selected from the group consisting of a ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift, and a chemical shift in said other dimension selected from the group consisting of ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift.

Also provided by the invention is a method of wherein said comparing step of step e) comprises comparing first one- or two-dimensional NMR spectra and second one- or two-dimensional NMR spectra after one or more said incubation times.

A method is provided wherein the determining step of step e) comprises a method selected from the group consisting of an algorithm, a computer algorithm, and visual inspection.

Another method of the invention provides a method wherein an interaction is selected from the group consisting of molecule-molecule binding, ligands bound to an enzymatic site of said target molecule.

In a further embodiment of this invention, it can be used in the presence of a first ligand for screening for ligands which exhibit highly desirable synergistic effects with the first ligand. Typical applications are for screening for ligands of enzymes which have more than two binding sites (substrate- and co-enzyme-binding sites, for instance) or ligands of proximal subsites on an enzyme with only one active center. Figure 3 depicts the reaction scheme when two substrate-competitive inhibitors (I_1 and I_2) and a substrate (S) react with an enzyme (E). This is well-known in the art of enzymology and was first demonstrated by Slater and Bonner, *Biochem. J.* 52 185 (1952). The initial steady-state rate equation for this system is expressed by:

$$v = V_m / \left[1 + \left(\frac{K_M}{S} \right) \left(1 + \frac{I_1}{K_{EI1}} + \frac{I_2}{K_{EI2}} + \frac{I_1 I_2}{\alpha K_{EI1} K_{EI2}} \right) \right]$$

where S, I₁ and I₂ are the substrate, inhibitor I₁ and inhibitor I₂ concentrations, respectively. An interaction constant α between I₁ and I₂ in the EI₁I₂ complex has the following meaning: when I₁ and I₂ interact with the same site of E, they prevent each other from binding to E, i.e. EI₁I₂ is not formed and thus $\alpha = \infty$. If I₁ and I₂ interact with different sites of E, $\infty > \alpha > 0$. When the interactions of I₁ and I₂ with E are strictly independent of each other, $\alpha = 1$. When a positive attraction occurs or if synergy exists between the two inhibitors in the EI₁I₂ complex, $1 > \alpha > 0$. When I₁ and I₂ interact repulsively in the EI₁I₂ complex, $\infty > \alpha > 1$. A number of causes may be involved in this interaction constant α , such as ion-dipole, interionic, interdipole, hydrophobic, and hydrophilic interactions, as well a steric hindrance and protein conformational changes. For screening for ligands in proximal subsites, this system is particularly useful since it provides a way to determine whether two ligands in proximal subsites form a suitable pair of ligands which can then be chemically combined to span the active site and increase affinity, as described in patent documents US5698401, US5804390, US5891643. For screening purposes in the presence of a first ligand, the interaction constant " α " serves merely to identify ligands that may potentially exhibit desirable synergistic effects with the first ligand. A preferred embodiment of the invention is based on a model that assumes that the inhibitors are substrate-competitive inhibitors. However, the modes of inhibition of the identified pairs of ligands are not necessarily known initially, and should not be deemed to be in any way limitative of the scope of the invention. Therefore, it is usually desirable to determine their mode of inhibition by standard enzymatic methods, such as the Lineweaver-Burks method, among others, in order to further characterize them and ascertain their relevance in a given situation. The skilled artisan will understand what method to use in a given situation in light of the disclosure herein and the state of the art. One preferred embodiment of this method comprising the steps of: a) exposing a substrate to a target molecule for one or more incubation times; b) generating a first spectrum that displays either a chemical shift in the first dimension or a chemical shifts in an other dimension of the substrate or product in step a) that has been exposed to the target molecule; c) mixing a substrate or product with the first ligand; d) exposing the substrate or product and the first ligand to the target molecule for one or more incubation times; e) generating a second spectrum that displays either a chemical shift in the first dimension or a chemical shift in the other dimension of substrate or product in step c) that has been exposed to the target molecule

in step d) in the presence of the first ligand in step c); f) mixing the substrate or product with one or more chemical compounds; g) exposing the substrate or product and one or more chemical compounds to a target molecule for one or more incubation times; h) generating a third spectrum that displays either a chemical shift in the first dimension or a chemical shift in the other dimension of substrate or product in step f) that has been exposed to the target molecule in step g) in the presence of the one or more chemical compounds in step f); i) mixing substrate or product with first ligand and one or more chemical compounds; j) exposing the substrate or product, the first ligand and the one or more chemical compounds to the target molecule for one or more incubation times; k) generating a fourth spectrum that displays either a chemical shift in the first dimension or a chemical shift in the other dimension of the substrate or product in step i) that has been exposed to the target molecule in step j) in the presence of the first ligand and the one or more chemical compounds in step f); l) determining a conversion rate or conversion rates of each substrate or product from each spectrum of steps b), e), h) and k); and deriving an interaction constant (α) from a steady-state rate equation.

Targets, products, ligands and substrates of the invention may be polypeptides and/or polynucleotides. These compounds may be natural substrates, products and ligands or may be structural or functional mimetics. See, e.g., Coligan et al., Current Protocols in Immunology 1(2): Chapter 5 (1991).

Moreover, these compounds may be polypeptides and polynucleotides responsible for many biological functions, including many disease states from which an individual may suffer, in particular human diseases. It is therefore desirable to devise screening methods to identify compounds which stimulate or which inhibit the function of a polypeptide or polynucleotide, and such methods are provided herein. Compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. Such compounds include, for instance, agonists, antagonists or inhibitors that may be natural or modified substrates, products, ligands, receptors, enzymes, etc., as the case may be, or may be structural or functional mimetics thereof (see Coligan et al., Current Protocols in Immunology 1(2):Chapter 5 (1991)).

Fusion proteins, such as those made from Fc portion and a polypeptide can also be used for high-throughput screening assays to identify antagonists of a target polypeptide (see D. Bennett et al., J Mol Recognition, 8:52-58 (1995); and K. Johanson et al., J Biol Chem, 270(16):9459-9471 (1995)).

Certain methods of the invention are provided based on high throughput or parallel processing formats. One preferred high throughput embodiment is a methods wherein at least one chemical compound, but preferably a chemical mixture, is provided in a multiwell vessel loaded with target and ligand and/or substrate and/or product. The target may be
5 already present in each well prior to loading of the ligand and/or substrate and/or product, or may be simultaneously or subsequently added with the ligand and/or substrate and/or product. Mutli-well plates are well known, commercially available, and useful with the methods of the invention. Once the target, at least one chemical compound, and a ligand and/or substrate and/or product is in the well, the reactions is allowed to progress for some
10 desired time. After such time, the reaction is slowed or stopped (herein "quenched") using a suitable method, readily determinable by a skilled artisan or known in the art for a given target. For example, quenching may be affected by heating, freezing, or by the addition of a chemical compound, as illustrated in Figure 5. Common quenching compounds include, for example, alkylating agents, compounds that affect covalent modification of the target, target
15 active site binding agents, antibodies, ligand or substrate mimetics, salts, organic solvents, EDTA, sodium azide, among others. Following quenching a reaction, the samples are loaded into an NMR device. This loading can be performed manually or using a robot. In preferred embodiments each of the wells in a multiwell format is loaded with one or more compounds, an invariant concentration of substrate and/or ligand and/or product, and an
20 invariant concentration of target, most preferably an enzyme, either of protein or a ribozyme. In a preferred embodiment each reaction in the multiwell format is quenched simultaneously. This allows for each sample in each well to be loaded on an NMR device serially without having the undesirable effect of having reactions progress for different times. In another preferred embodiment, each or a series of reactions in the wells is
25 quenched at differing time points. For instance, one or more reactions, each with a different chemical compound or compound mixture, is stopped at a first time, followed by one or more reactions, each with a different chemical compound or compound mixture, is stopped at a second time, etc., for as many time points as is desired. It is preferred that the mixed reagents in reactions stopped at one time point are provided in duplicate or further
30 replicates and are be stopped at other time points. Two or more time points allow for one to calculate the effect of the chemical compound or mixture on the reaction over time. See, for example, Figure 4, which also relates to determination of the interaction constant. Moreover, Figure 6 illustrates a preferred embodiment of calulating the interaction constant using a graphical method with more measurements (See also, Yonetani, *et al.* (1982) Meth.

WO 01/23889

PCT/US00/26949

Enzymol. 87: 500-509, and Yonetani, *et al.* (1964) Arch. Bioch. Biophys. 106: 243, for methods of calculating the interaction constant).

Ligands and/or substrates and/or products useful in methods of the invention include, for example, small organic molecules, peptides, polypeptides and antibodies that bind to a polynucleotide and/or polypeptide and thereby inhibit or extinguish its activity or expression. Ligands and/or substrates and/or products useful in methods of the invention include, for example, small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that binds the same sites on a target molecule thereby preventing the action or expression of target polypeptides and/or polynucleotides by excluding target polypeptides and/or polynucleotides from binding.

Ligands and/or substrates and/or products useful in methods of the invention include, for example, a small molecule that binds to and occupies the binding site of a target polypeptide thereby preventing binding to cellular binding molecules, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, peptides or peptide-like molecules. Other potential antagonists include antisense molecules (see Okano, J. Neurochem. 56: 560 (1991); OLIGODEOXYNUCLEOTIDES AS ANTISENSE INHIBITORS OF GENE EXPRESSION, CRC Press, Boca Raton, FL (1988), for a description of these molecules). Preferred potential antagonists include compounds related to and variants of a target.

Other examples of potential polypeptide antagonists include antibodies or, in some cases, oligonucleotides or proteins which are closely related to the ligands, substrates, products, receptors, enzymes, etc., as the case may be, of a target, e.g., a fragment of the ligands, substrates, products, receptors, enzymes, etc.; or small molecules which bind to a target but do not elicit a response, so that the activity of the target is prevented.

GLOSSARY

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"Antibody(ies)" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library.

"Bodily material(s)" means any material derived from an individual or from an organism infecting, infesting or inhabiting an individual, including but not limited to, cells, tissues and

WO 01/23889

PCT/US00/26949

waste, such as, bone, blood, serum, cerebrospinal fluid, semen, saliva, muscle, cartilage, organ tissue, skin, urine, stool or autopsy materials.

"Fusion protein(s)" refers to a protein encoded by two, often unrelated, fused genes or fragments thereof. In one example, EP-A-0464 discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, employing an immunoglobulin Fc region as a part of a fusion protein is advantageous for use in therapy and diagnosis resulting in, for example, improved pharmacokinetic properties [see, e.g., EP-A 0232262]. On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified.

"Individual(s)" means a multicellular eukaryote, including, but not limited to a metazoan, a mammal, an ovid, a bovid, a simian, a primate, and a human.

"Isolated" means altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Moreover, a polynucleotide or polypeptide that is introduced into an organism by transformation, genetic manipulation or by any other recombinant method is "isolated" even if it is still present in said organism, which organism may be living or non-living.

"Polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotide(s)" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded regions, or a mixture of single- and double-stranded regions. In addition, "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other

reasons are "polynucleotide(s)" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells. "Polynucleotide(s)" also embraces short polynucleotides often referred to as oligonucleotide(s).

"Polypeptide(s)" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" refers to both short chains, commonly referred to as peptides, oligopeptides and oligomers and to longer chains generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993) and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in POSTTRANSLATIONAL

WO 01/23889

PCT/US00/26949

COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter et al., Meth. Enzymol. 182:626-646 (1990) and Rattan et al., Protein Synthesis: Posttranslational Modifications and Aging, Ann. N.Y. Acad. Sci. 663: 48-62 (1992). Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and
5 branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

"Variant(s)" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference
10 polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusion proteins and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference
15 polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. The present invention also
20 includes include variants of each of the polypeptides of the invention, that is polypeptides that vary from the referents by conservative amino acid substitutions, whereby a residue is substituted by another with like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly
25 preferred are variants in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acids are substituted, deleted, or added in any combination. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods
30 known to skilled artisans.

EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1 Deformylation by Peptide Deformylase

In a preferred embodiment of the present invention, catalytic efficiency is exploited in order to decrease the amount of target molecule required to detect binding of a compound to the target molecule. One may readily monitor changes in the conversion rate of a substrate into a product when the compound is present. Figures 1 and 2 illustrate such changes observed in the rate of deformylation of For-Met-Ala-Ser-OH by Peptide Deformylase (S.Aureus) when 8-hydroxyquinoline is present in the solution. With a catalytic efficiency in the range of $10^5 \text{ s}^{-1}\text{M}^{-1}$ for that substrate, it is possible to use a target concentration which is at least two orders of magnitude smaller than the concentration that would have been required for identifying 8-hydroxyquinoline as a ligand by the well-known NMR differential line broadening technique. Staphylococcus aureus Peptide Deformylase is described in U.S. Patent Application Serial Number 08/911,844, filed August 15, 1997, and and European Patent Application EP0879879 A2 and EP00879879A3. The methods of the example could also be adapted for use with Streptococcus pneumoniae pdf1 (U.S. Patent Application Serial Number 08/991,023, filed December 15, 1997 and European Patent Applications EP00863152A3 and EP00863152A2) or Streptococcus pneumoniae pdf2 (U.S. Patent Application Serial Number 08/989,558, filed December 12, 1997 and European Patent Application EP00863205A1).

What is claimed is:

1. A method of identifying compounds that interact with a target molecule comprising the steps of:

a) mixing a substrate, product or ligand of a target with at least one chemical compounds;

5 b) generating a first spectrum that displays either a chemical shift in the first dimension or a chemical shifts in the other dimension of substrate, product or ligand in step a);

c) exposing substrate, product or ligand and mixture of chemical compounds in step a) to a target molecule for one or more incubation times;

10 d) generating a second spectrum that displays either a chemical shifts in the first dimension or a chemical shifts in the other dimension of substrate or product in step a) that has been exposed to the target molecule in step c) in the presence of one or mixture of chemical compounds in step a);

e) comparing said first spectrum and second spectrum after one or more said incubation times in step c) to determine at least one difference between said first spectrum and second spectrum, the differences observed along either or both chemical shift dimensions identifying the transformation of said substrate and classifying the presence of one or more compounds that are substrates, products or ligands that interact with said target molecule.

15 2. The method of claim 1 wherein step a) further comprises a target that is a biomolecule.

3. The method of claim 1 wherein step a) further comprises a chemical compound that is in solution or attached to a solid substrate or matrix.

20 4. The method of claim 1 wherein step b) further comprises a first spectrum selected from the group consisting of a one-dimensional, two-dimensional or three-dimensional spectrum.

5. The method of claim 4 wherein said first spectrum displays a chemical shift in said first dimension selected from the group consisting of ¹H, ³H, ¹¹B, ¹³C, ¹⁵N, ¹⁹F, ²⁹S or ³¹P chemical shift, and a chemical shift in said other dimension selected from the group consisting of ¹H, ³H, ¹¹B, ¹³C, ¹⁵N, ¹⁹F, ²⁹S or ³¹P chemical shift.

25 6. The method of claim 1 wherein said exposing step of step c) further comprises a mixture comprising between 2 and 100 chemical compounds.

WO 01/23889

PCT/US00/26949

7. The method of claim 1 wherein said incubation times number between 1 and 20, 30, 40, 50 or greater.
8. The method of claim 1 wherein step d) said second spectrum displays a chemical shift in said first dimension selected from the group consisting of
5 ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift, and a chemical shift in said other dimension selected from the group consisting of ^1H , ^3H , ^{11}B , ^{13}C , ^{15}N , ^{19}F , ^{29}S or ^{31}P chemical shift.
9. The method of claim 1 wherein said comparing step of step e) comprises comparing first one- or two-dimensional NMR spectra and second one- or two-dimensional NMR spectra after one or more said incubation times.
10. The method of claim 1 wherein the determining step of step e) comprises a method selected from the group consisting of an algorithm, a computer algorithm, and visual inspection.
11. The method of claim 1 wherein an interaction is selected from the group consisting of molecule-molecule binding, ligands bound to an enzymatic site of said target molecule.
12. A method of determining an interaction constant (α) comprising the steps of:
a) exposing a substrate or product to a target molecule for one or more incubation times; b)
10 generating a first spectrum that displays either a chemical shift in the first dimension or a chemical shifts in an other dimension of the substrate or product in step a) that has been exposed to the target molecule; c) mixing a substrate with the first ligand; d) exposing the substrate and the first ligand to the target molecule for one or more incubation times; e)
15 generating a second spectrum that displays either a chemical shift in the first dimension or a chemical shift in the other dimension of substrate or product in step c) that has been exposed to the target molecule in step d) in the presence of the first ligand in step c); f) mixing the substrate or product with one or more chemical compounds; g) exposing the substrate or product and one or more chemical compounds to a target molecule for one or more incubation times; h) generating a third spectrum that displays either a chemical shift in the first
20 dimension or a chemical shift in the other dimension of substrate or product in step f) that has been exposed to the target molecule in step g) in the presence of the one or more chemical compounds in step f); i) mixing substrate or product with first ligand and one or more chemical compounds; j) exposing the substrate or product, the first ligand and the one or more chemical compounds to the target molecule for one or more incubation times; k) generating a
25 fourth spectrum that displays either a chemical shift in the first dimension or a chemical shift

in the other dimension of the substrate or product in step i) that has been exposed to the target molecule in step j) in the presence of the first ligand and the one or more chemical compounds in step f); l) determining a conversion rate or conversion rates of each substrate or product from each spectrum of steps b), e), h) and k); and deriving an interaction constant (α) from a steady-state rate equation.

13. A method using NMR for of screening for ligands which exhibit synergistic effects on a target in the presence of another ligand.

14. The method of claim 12 wherein the target is an enzyme comprising more than two binding sites.

15. The method of claim 14 wherein the two binding sites are a substrate- and co-enzyme-binding site.

16. The method of claim 12 wherein the rate is determined by the following equation:

$$v = V_m / \left[1 + \left(\frac{K_M}{S} \right) \left(1 + \frac{I_1}{K_{EI1}} + \frac{I_2}{K_{EI2}} + \frac{I_1 I_2}{\alpha K_{EI1} K_{EI2}} \right) \right]$$

wherein S, I_1 and I_2 are the substrate, inhibitor I_1 and inhibitor I_2 concentrations, respectively.

17. The method of claim 1 wherein said at least one chemical compound is provided in a multiwell vessel loaded with target and substrate, ligand, or product.

18. The method of claim 17 wherein a target-substrate reaction is quenched at a selected time.

19. A method of identifying compounds that interact with a target molecule comprising the steps of:

- a) exposing substrate to a target molecule for one or more incubation times;
- b) generating one or more spectra at one or more incubation times of said substrate and said target molecule of step a),
- c) exposing said substrate and one or mixture of chemical compounds for one or more incubation times;
- d) generating one or more spectra at one or more incubation times of said substrate, said target molecule and said compounds of step c);
- e) comparing at least one spectrum of step b) with at least one spectrum of step d) to determine at least one difference between said spectrum of step b) with said spectrum of step d), the differences observed along either or both chemical shift dimensions identifying the

WO 01/23889

PCT/US00/26949

transformation of said substrate and classifying the presence of one or more compounds that are substrates, products or ligands that interact with said target molecule.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number
WO 01/23889 A1

(51) International Patent Classification⁷: **G01N 33/53**,
33/557, 33/566, 33/567, 24/00, G01R 33/035

[US/US]; 22 Greythorne Woods Circle, Wayne, PA 19087 (US).

(21) International Application Number: PCT/US00/26949

(74) Agents: **GIMMI, Edward, R.** et al.; SmithKline Beecham Corporation, Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).

(22) International Filing Date:
29 September 2000 (29.09.2000)

(25) Filing Language: English

(81) Designated States (*national*): CA, JP, US.

(26) Publication Language: English

(84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

(30) Priority Data:
60/156,557 29 September 1999 (29.09.1999) US

Published:

- With international search report
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

(71) Applicant (*for all designated States except US*):
SMITHKLINE BEECHAM CORPORATION
[GB/GB]; One Franklin Plaza, Philadelphia, PA 19103 (GB).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): **BRIAND, Jacques**

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette

(54) Title: METHOD OF USING ONE-DIMENSIONAL AND MULTI-DIMENSIONAL NUCLEAR MAGNETIC RESONANCE TO IDENTIFY COMPOUNDS THAT INTERACT WITH TARGET BIOMOLECULES

(57) Abstract: The invention provides a method using one-dimensional and multi-dimensional NMR spectroscopy for identifying ligands to target biomolecules.

WO 01/23889 A1

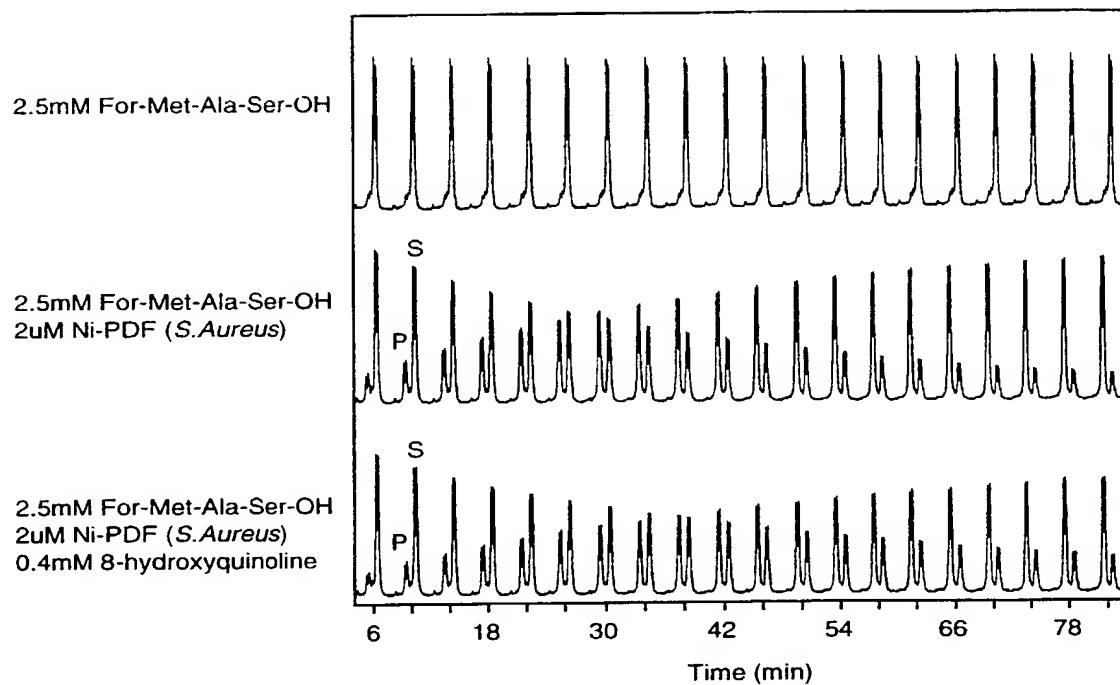
5006 833 V.P. OFFICE, 1/1/11

10/070128

WO 01/23889

PCT/US00/26949

Figure 1



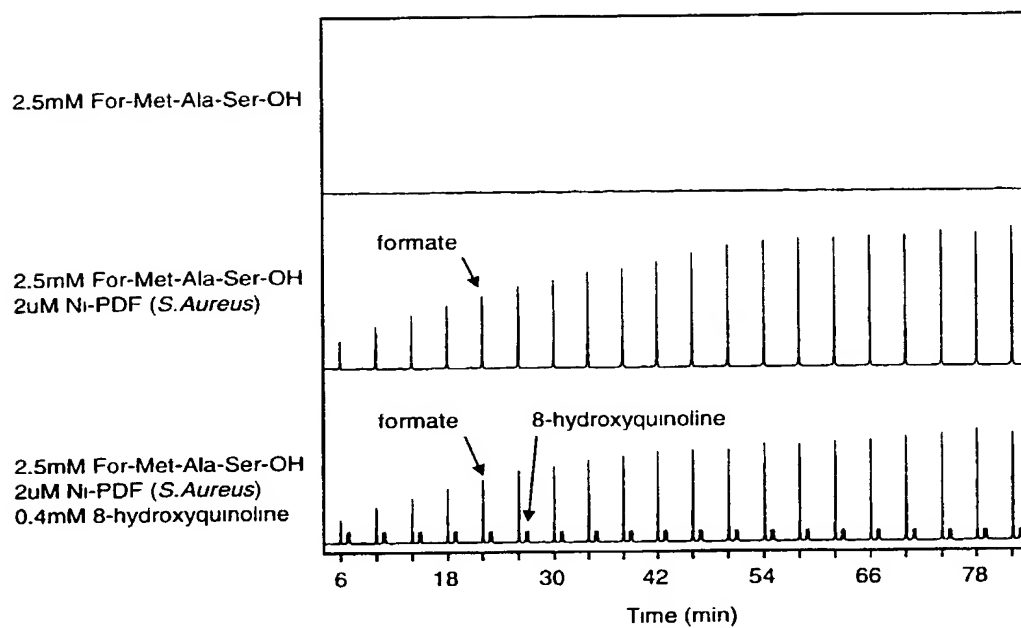
4-11-94

10/070128

WO 01/23889

PCT/US00/26949

Figure 2



10/070128

WO 01/23889

PCT/US00/26949

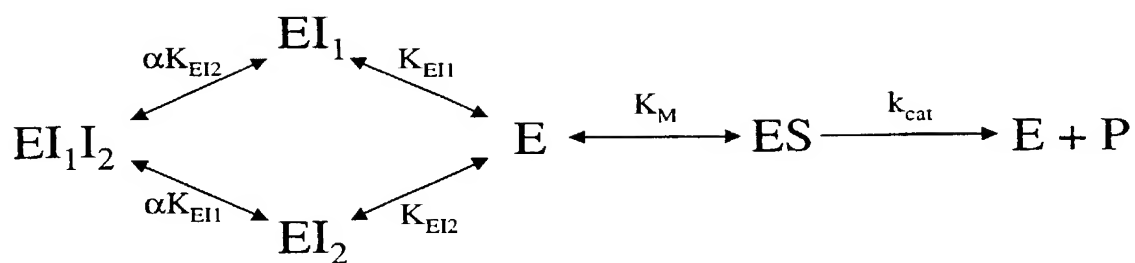


Figure 3

10/070128

WO 01/23889

PCT/US00/26949

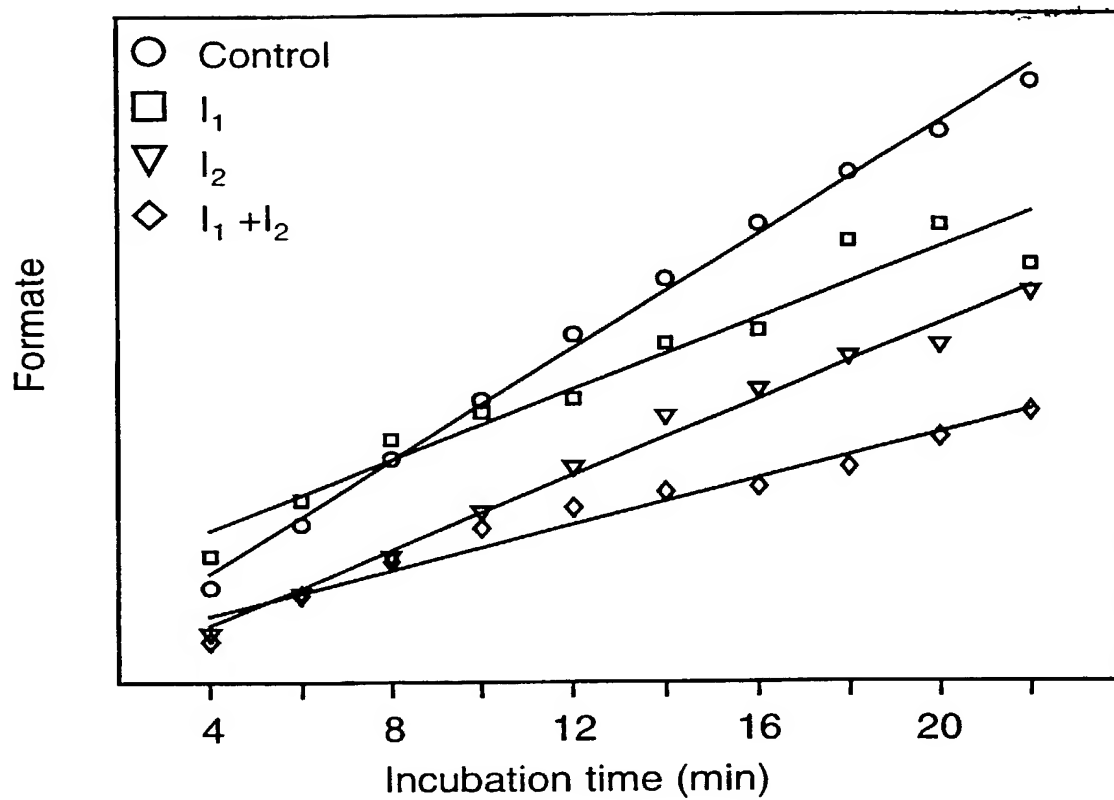
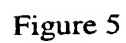


Figure 4

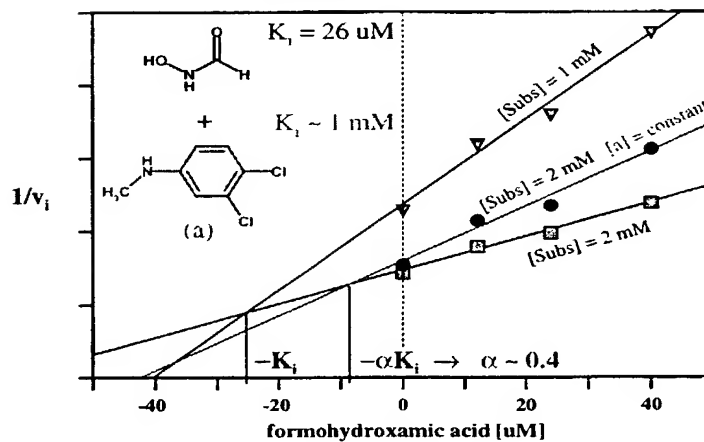
WO 01/23889

PCT/US00/26949

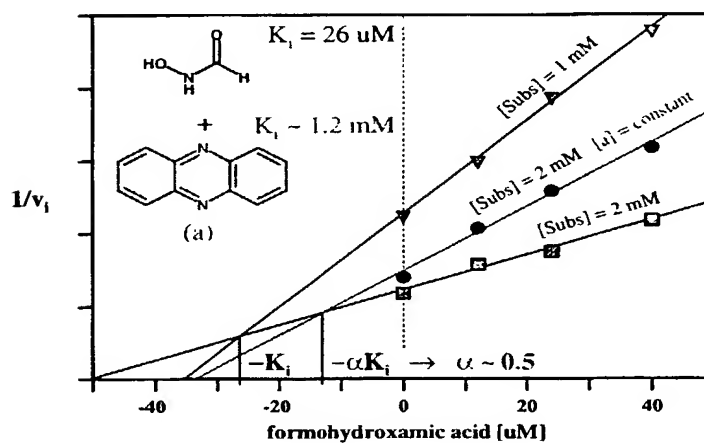


a)

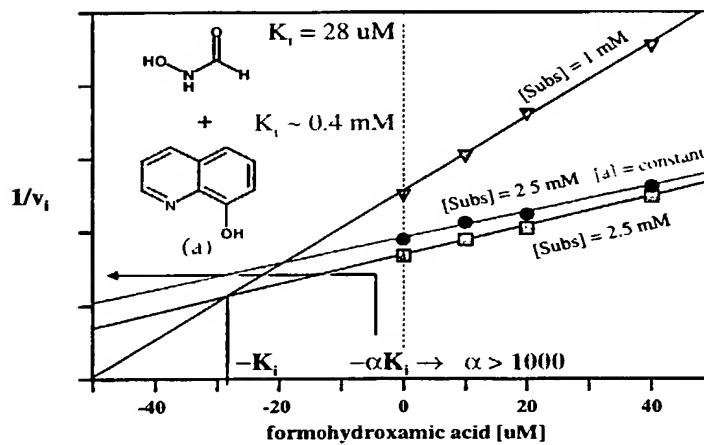
Figure 6



b)



c)



Docket No.: P51032

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Method of Using One-Dimensional and Multi-Dimensional Nuclear Magnetic Resonance To Identify Compounds That Interact With Target Biomolecules

the specification of which (check one)

☐ is attached hereto.

☒ was filed on **29 September 2000** as Serial No. **PCT/US00/26949**
and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or Inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed
--------	---------	-------------	------------------

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date
60/156,577	29 September 1999

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the

filing date of the prior application and the national or PCT international filing date of this application.

Serial No.	Filing Date	Status
------------	-------------	--------

I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

Customer Number 20462.

Address all correspondence and telephone calls to Edward R. Gimmi, SmithKline Beecham Corporation, Corporate Intellectual Property-U.S., UW2220, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939, whose telephone number is 610-270-4478.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00 Full Name of Inventor: Jacques BRIAND

Inventor's Signature: Jacques Briand

Date: 01/23/01

Residence: 22 Greythorne Woods Circle, Wayne, Pennsylvania 19087

Citizenship: Canada

Post Office Address: SmithKline Beecham Corporation
Corporate Intellectual Property - UW2220
P.O. Box 1539
King of Prussia, Pennsylvania 19406-0939

PA